GENETIC RECOMBINATION IN DNA-INDUCED TRANSFORMATION OF PNEUMOCOCCUS. I. THE PROBLEM OF RELATIVE EFFICIENCY OF TRANSFORMING FACTORS[1]

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FOLLOWING absorption of DNA by competent cells, genetic transformation ensues with a probability that is characteristic for each particular genetic marker (HOTCHKISS and MARMUR 1954; LERMAN and TOLMACH 1957). Thus, *specific incorporation*, defined as the amount of DNA which must be incorporated in order to obtain one transformant, varies according to the genetic marker employed.

With the development of more elaborate genetic marker systems, notably, systems of linked genes, various causes were postulated as operating in determining the probability that, given DNA incorporation, a certain type of transformant would appear. Thus, arguing from the fact that pairs of linked genes appear together in transformants less often than the same genes appear singly (HOTCHKISS and EVANS 1958), it has been suggested that mutant markers which have different probabilities of integration into a transformed cell have different linear dimensions (LACKS and HOTCHKISS 1960; EPHRUSSI-TAYLOR 1961).

In an interesting series of experiments, SCHAEFFER (1958) showed that where transformations are performed between different species of Hemophilus, relatedness, which is presumably an expression of homology of base-pair sequence in DNA, is crucial in determining the probability of integration of a genetic marker into a transformant. Further, in intraspecific transformation in pneumococcus, GREEN (1959) found that certain combinations of donor and recipient led to low transformation frequencies for a particular streptomycin resistance gene. The depressed probability of integration was further shown to be due to the presence of a "depressor" region closely associated with the marker gene, but separable from it by recombination (GREEN 1959; ROTHHEIM 1962). Both of these lines of work indicate the importance of structural homology in determining the probability of integration.

Still another cause of low probability of genetic integration has been proposed, though never demonstrated experimentally. Were a particular genetic marker to be located always near the end of a DNA molecule, one might expect it to be

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recombined into a transformant with a low probability (EPHRUSSI-TAYLOR 1961). This suggestion is based on a particular model of the recombination process, and it should be noted that other models could lead to the opposite prediction. For example, if recombination requires strand separation of the donor DNA, markers near ends might participate more actively in the recombination process owing to easier strand separation.

Finally, with the discovery that physical uptake of DNA by competent cells is molecular-weight dependent (ROSENBERG, SIROTNAK and CAVALIERI, 1959; LITT 1958), in that larger molecules are more readily absorbed, one could suppose that different markers, upon extraction, are associated with DNA fragments of different size. Hence, different markers could exhibit different probabilities of integration, owing to selective absorption of the larger fragments (MARMUR, ANDERSON, MATHEWS, BERNS, GAJEWSKA, LANE and DOTY 1961).

Distinguishing between these various possible factors requires primarily that the experimenter be in possession of a system of genetic analysis with which fine-structure studies can be performed. Given a single densely marked region of a DNA molecule, the majority of these possible causes of high or low efficiency can be tested. Having developed such a system (SICARD 1964), we have undertaken to examine what determines the transforming efficiency of the mutant markers in the densely marked region.

The experiments to be described here concern 73 mutants, 39 of which have been mapped in 30 sites, within what appears to be a single functional unit of the pneumococcal genome (SICARD 1964). These sites are all genetically linked (SICARD and EPHRUSSI-TAYLOR, in preparation). All mutants in this region are resistant to aminopterin, and sensitive to an imbalance in the molar concentrations of isoleucine, leucine and valine. Thus, transformations can be selectively scored in crosses of wild-type cells by mutant DNA, or mutant cells by wild-type DNA. Owing to the special advantages of this genetic system, it will be shown below that the probability of genetic integration of these particular markers is determined by highly local factors; i.e., either by the nature of the mutation, or by the base composition of the region in which a mutation has occurred.

MATERIALS AND METHODS

Media, transformation techniques and strains employed in our laboratory have been recently described (SICARD 1964). Pertinent to what follows is the fact that all mutants have been isolated within a single wild-type line, to render genetic homology as great as possible. Competence is obtained by transfer of a small aliquot of frozen stock culture (preculture, or PC) into the appropriate medium, where it develops in an acute fashion at a predictable time after transfer. Since competence appears and disappears in an interval of 10 to 15 minutes, it is not necessary to arrest the reaction of cells with DNA by the addition of DNase. Variability in transformation could be expected to occur because of slight physiological differences in batches of PC, or in batches of competent cells made from the same PC. The incidence of such variability on estimations of transforming efficiency will be examined below. By "complexes" we mean cells which have fixed DNA irreversibly, but which are not yet transformed.

Mutants induced with ethyl methane sulfate (EMS) were obtained by growing a streptomycin-resistant transformant of the wild-type line in 0.5% EMS for about six generations, and plating in 1×10^{-5} aminopterin.

All mutants having the dual property of resistance to 1×10^{-5} M aminopterin, and sensitivity to an imbalance of branched amino acids, map in a single region of a DNA molecule and are presumed to be in a single cistron referred to as the *amiA* cistron. Mutants bearing the numbers r1 and r2, r21 through r40, and above r53 are of spontaneous origin. Mutants numbered from r3 through r20 are HNO_2-induced (technique of LITMAN and EPHRUSSI-TAYLOR 1959), while r41 through r53 are EMS-induced.

Ultraviolet irradiations were carried out on dilute solutions of DNA (about 10 μg/ml), using a lamp emitting light at 257 mμ, at a dose rate of 17.1 ergs per sec per mm^2 (Black Light Eastern Corp., model R-57). Solutions were stirred during irradiation.

Statistical analyses were carried out according to KIMBALL (1961), who has treated specifically the problem of evaluating the errors in measuring ratios. DR. NORMAN RUSHFORTH has been of invaluable assistance in adapting KIMBALL's method to the present data.

EXPERIMENTAL RESULTS

The efficiency of the amiA *mutants.* This is determined as a ratio; i.e., the number of transformants of a particular type, relative to the number of transformants for a standard reference gene, *str-r41*. The reference gene serves to measure, essentially, the amount of DNA fixed by a particular lot of cells. This practice is widely used in place of measuring P^{32} incorporation since it is more convenient, and avoids the problem of radiation damage to the DNA (GREEN 1959; LACKS and HOTCHKISS 1960; EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; ANAGNOSTOPOULOS and CRAWFORD 1961). The distribution of the efficiencies of the 73 *amiA* mutants is shown in Figure 1. In our experience, these values are reproducible, and variability lies within the error of the plating tech-

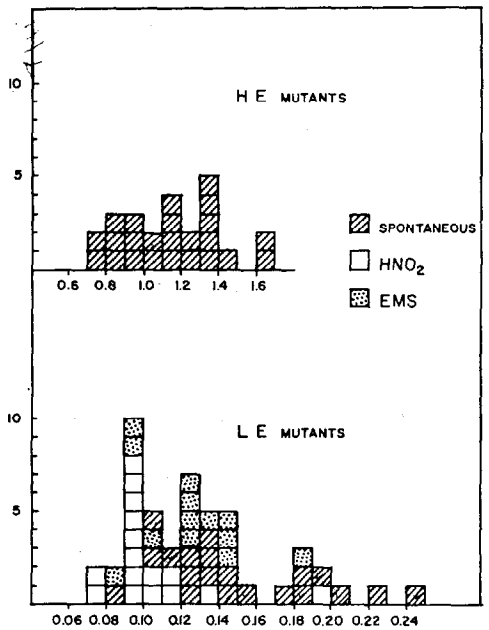

FIGURE 1.—Frequency distributions of transforming efficiencies of mutants selected in the *amiA* locus.

nique. This is in contrast with the experience of IYER and RAVIN (1962) working with several erythromycin resistance factors, who found considerable variation depending on the batch of recipient cells employed. The efficiency measurements performed on eight amylomaltase marker genes by LACKS and HOTCHKISS (1960) also show in several instances greater standard deviations than we have found. On the other hand, relative constancy of efficiency determinations has been noted by SIROTNAK, LUNT and HUTCHINSON (1964) for a series of amethopterin resistant mutants in pneumococcus. However, the methods of statistical evaluation employed in the above-mentioned studies have not been indicated, so that it is impossible to compare published data in a significant way.

The first 30 mutants, and many of the remaining 43, have been used not only as DNA donors in one-point crosses of wild-type recipient by *amiA-r str-r41* DNA, but also as recipients in one-point crosses of *amiA-r* cells by *amiA-s str-r41* DNA. With the possible exception of mutant *r30*, efficiency for any given mutant is the same in both directions of the one-point crosses. Mutant *r30* usually gives a value of about 0.6 in the cross of mutant cells by wild-type DNA, and about 0.8 in the reverse cross. In paired experiments done the same day, this difference in the two directions of one-point crossing of site 30 has been found to be significant at the 5% level. Since each cross to be described in the report to follow the present one (on mapping of the genes) includes a control cross of mutant recipient by wild-type (for *amiA*) donor DNA, more information is available on the reproducibility of the efficiency measurement in the one-point transformation cross of mutant to wild type. Table 1 presents data from independent experiments of this type, demonstrating the constancy of efficiency.

The striking feature of the data of Figure 1 and Table 1 is that mutants within the *amiA* region fall into two nonoverlapping classes with respect to efficiency. These classes will be called HE and LE classes, for high and low efficiency.

Relationship between efficiency of a mutant and its origin: The distribution of the 73 *amiA-r* mutants into two efficiency classes relative to origin of the mutant is shown in Table 2. Spontaneous mutation gives rise to the two classes with roughly equal frequency, whereas EMS has given rise only to LE mutants. Of the 18 mutants isolated following transformation of wild-type cells with HNO_2-treated wild-type DNA (see SICARD 1964), only two are HE (*r3* and *r19*). Since mutagenesis by transformation of cells with HNO_2-treated DNA increases the incidence of *amiA* mutants by a factor of 10 relative to background mutation, on the average one out of every ten presumably induced mutants could in fact be of spontaneous origin. Both of the HE mutants isolated in the HNO_2-induced group fail to show recombination with spontaneous mutants *r1* and *r2* which themselves do not recombine, thus marking a "hot-spot" of spontaneous mutation. This suggests that mutants *r3* and *r19*, of the HNO_2 group, are in reality of spontaneous origin. A further reason for believing that HNO_2 induces only LE mutants is the fact that the very process by which these mutants are obtained would favor the detection of HE mutants. The HNO_2-altered DNA is used as a transforming factor, and HE induced mutations would have a ten times greater chance of appearing in transformants than would LE mutations. There

TABLE 1

Statistical variability of efficiency measurements

Cross	PC No.	Complex No.	Ratio	Limits
amiA-r1 × wild type	1	1	1.05	0.93 – 1.19
	2	1	1.21	1.05 – 1.38
amiA-r9 × wild type	1	1	0.082	0.072 – 0.101
	1	2	0.086	0.075 – 0.095
amiA-r15 × wild type	1	1	0.0936	0.082 – 0.107
amiA-r16 × wild type	1	1	0.116	0.105 – 0.128
	1	2	0.122	0.112 – 0.134
	2	1	0.111	0.102 – 0.120
ami-17 × wild type	1	1	0.089	0.072 – 0.098
	1	2	0.093	0.083 – 0.101
	2	1	0.104	0.098 – 0.111
	2	2	0.133	0.117 – 0.152
ami-22 × wild type	2	2	1.13	1.05 – 1.22
	2	3	1.01	0.92 – 1.10
	2	4	0.915	0.84 – 1.00
ami-29 × wild type	1	1	1.03	0.96 – 1.11
	1	1 (bis)	0.88	0.80 – 0.96
	1	2	1.03	0.94 – 1.13
ami-30 × wild type	1	1	0.634	0.589 – 0.683
	1	1 (bis)	0.696	0.646 – 0.749
	1	2	0.727	0.659 – 0.805
	1	3	0.664	0.597 – 0.738
	2	1	0.667	0.653 – 0.702

Values are included which were obtained (a) in different platings of same batch of DNA-bacterial complexes; (b) in different preparations of complexes from a single preculture; (c) in completely independent experiments from different lots of frozen preculture. PC refers to batch of preculture. Variability is expressed as upper and lower limits of the 95% confidence interval.

TABLE 2

Incidence of HE and LE mutants according to their origins

	Type of mutant	
	HE	LE
Spontaneous, lot 1	7	5
Spontaneous, lot 2	5	4
Spontaneous, lot 3	8	9
Spontaneous, lot 4	2	2
Total	22	20
percent	53	47
HNO_2-induced	2*	16
percent	0(11)	100(89)
EMS-induced	0	13
percent	0	100

* Probably of spontaneous origin.

is, therefore, strong reason to believe that treatment of DNA with HNO_2 yields only LE mutants.

Mapping the amiA *mutants:* The transforming efficiency of a mutant plays a very curious and important role in two-factor mapping experiments (LACKS and HOTCHKISS 1960), in which wild-type recombinants are scored in crosses of mutant by mutant. If a particular cross involves a pair of mutants which have different efficiencies, as defined above, the frequency of wild-type recombinants observed will very much depend upon whether the LE site is in the donor DNA, or the recipient cell. When the LE site is in the recipient cell, recombinants will be very much rarer. To counter this difficulty, LACKS and HOTCHKISS proposed that recombination values should be corrected by dividing the observed recombination value by the efficiency of the site present in the recipient cell. In spite of the fact that such presumed corrections can leave differences as great as threefold or more in recombination frequencies of crosses in reverse polarity, several attempts at mapping by this method have been published (EPHRATI-ELIZUR, SRINIVASIN and ZAMENHOF 1961; ANAGNOSTOPOULOS and CRAWFORD 1961; SIROTNAK, LUNT and HUTCHINSON 1964). The persistence of these differences demonstrates that there is no simple correlation between efficiency, in one-point transformations, and recombination frequency, in two-point transformations. This question will be treated *in extenso* in the next paper of this series, now in preparation. A further difficulty with the mapping method proposed by LACKS and HOTCHKISS is that suitable statistical methods to compare numbers which, each, are a ratio of two ratios have apparently not been developed.

The mutants which have been mapped at the *amiA* locus have been located with respect to each other on the basis of two sorts of evidence: (1) Some mutants do not recombine and are presumed to lie at the same site; (2) In a series of crosses in which a given mutant strain serves as recipient, the approximate alignment of all other genes is made on the basis of the relative frequencies of wild-type recombinants observed. The independently obtained alignments are then compared. It is found that a high degree of concordance is observed for sites that are relatively close together. Therefore, by varying the site in the recipient, it is possible to align sites at any point of the map. This method avoids assigning a specific meaning to the actual recombination values observed. Mutants can be ordered with a fair degree of confidence in this way, but distances between them remain unknown, except in a gross way. Mapping data will be presented in the next paper of this series, but the map itself must be presented here in order to discuss the efficiency problem (see Figure 2). Most of the mutants numbered from 31 through 73 have not been completely mapped as yet. They fall at a large number of new sites, and appear to extend map length by a factor of the order of 2.

Site *r30* has been indicated to be a multisite mutant, covering sites *r17* and *r5*. The reasons for doing this, instead of placing *r30* between 5 and *17*, are the following. Crosses of strain r17 by DNA of r5 yielded 9 *amiA-s* recombinants for 57,450 *str-r* transformants scored. Strain r30 crossed by DNA *r17* has shown no *amiA-s* recombinants for 258,150 *str-r* transformants scored; and crossed by

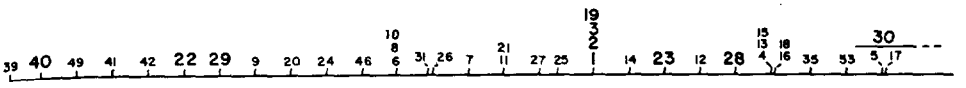

FIGURE 2.—Map of the genes whose positions have thus far been determined. Mutants which recombine with very low frequency are placed close together. Others are equally spaced, indicating that we cannot evaluate their relative distances with any degree of certainty. The order of the closely linked pairs of genes (*5* and *17*; *26* and *31*; *4* and *16*) may be the inverse of that shown.

DNA *r5*, no *amiA-s* recombinants for 207,000 *str-r* transformants scored. It is thus unlikely that *r30* lies between *r5* and *r17*. Further, mapping data show that *r30* is much closer to *r53* than is either *r17* or *r5*, indicating that *r30* extends appreciably to the left of the position occupied by *r17*. Figure 3 shows the recombination relationships of the mutants at the extreme right end of the map, in crosses with *r30* as recipient, with *r17* as recipient, and with *r53* as recipient.

Relationship between efficiency and position on the map: Inspection of the map shows two things. First, there is no over-all correlation between position on the map and efficiency. Second, with the exception of mutant *r30*, independent mutations at the same site are either all HE or all LE. The first observation

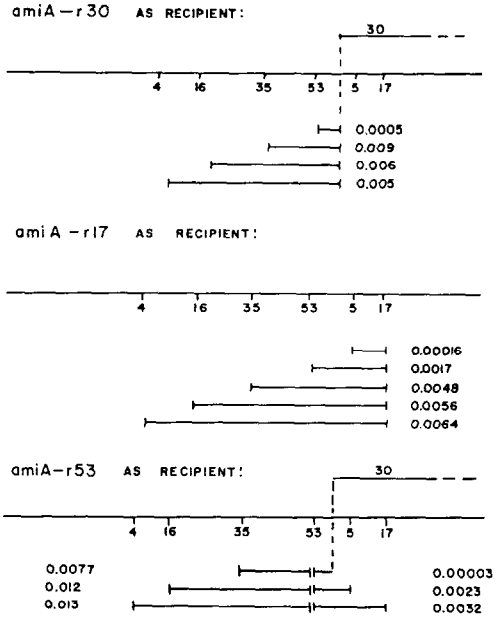

FIGURE 3.—Mapping the *r30* region, using strains r30, r17 and r53 as recipients. Recipients r17 and r53 would be expected to yield low frequencies of recombinants since *r17* and *r53* are LE genes. In spite of this, strain r30 × DNA *r53* gives fewer wild-type recombinants than strain r17 × DNA *r53*, indicating that mutation *r30* extends leftwards, ending close to site *r53*. This is consistent with the cross of strain r53 by DNAs *r30*, *r5* and *r17*, where *r30* also maps very much closer to site *r53* than do *r5* and *r17*. Gene order in crosses of recipient r30 is not the same as the order observed in crosses of recipients r17 and r53. The latter order is confirmed in crosses (not shown) of recipient r28, by all sites to its right. Site *r28* lies to the left of *r4*.

suggests that low efficiency has nothing to do with proximity of a site to the end of a molecule; were this the case, there should be a gradient with respect to efficiency. The second observation suggests that efficiency is site specific.

Mutant *r30* is a most interesting exception in that it is a mutant whose efficiency lies at the lower limit of the HE class, yet it covers two LE sites. We are searching for more mutants in this region of the map in an attempt to determine the length of mutation *r30*, and whether it covers HE sites as well as LE sites.

As the map stands at present, there is a strong indication that efficiency is site-specific and independent of the direction of the cross, and that it is not correlated with any major discontinuity; i.e., "end," either of the DNA molecule, or of the pairing process.

Differences in sizes of mutant markers as a cause of different efficiencies: A general correlation between efficiency, on the one hand, and linear dimensions of a mutation, on the other, was suggested by the study of LACKS and HOTCHKISS (1960). Of a group of eight mutants affecting a single enzyme, two mapped as small deletions (or multisite mutants), and one as a large deletion. Strains bearing these mutants could be transformed back to wild type, and the efficiency of such transformation appeared to be inversely correlated with the extent of the deletion. Furthermore, heating of wild-type DNA at temperatures causing depurinization but not strand separation resulted in an inactivation of the wild-type sites corresponding to the presumed size of the various mutations. In general, the larger the region required for transformation, as indicated by genetic data, the more rapid the inactivation proved to be.

We have considered whether our mutants differ in size, and whether such differences, if any, are correlated with efficiency. At present, appreciable size-differences seem excluded for the following reasons: (1) The *amiA* cistron is fairly intensely marked by the mutants thus far mapped, and only one has proven definitely to be multisite (*r30*). It is unlikely that many of the others represent gross mutational alterations. (2) In the course of the present study, spontaneous reversion has thus far been observed for the following mutants: HE—*r1*, *r22* and *r29*; LE—*r6*, *r8*, *r9* and *r10*. All revertants which have shown up have been examined for the presence of suppressor mutations by transforming wild-type cells with DNA of the revertants, and screening for the appearance of *ami-r* transformants. All revertants appear to be true reversions. We cannot, however, exclude the possibility of there being suppressors extremely closely linked to the original mutant site. As the reversion data stand they suggest that neither efficiency class has the reverse mutation pattern of a deletion or multisite mutation.

While there is no genetic evidence of gross size differences between HE as opposed to LE genes, one can nonetheless ask whether such differences do exist. Following LACKS and HOTCHKISS (1960), and ROGER and HOTCHKISS (1961), heat inactivation at subdenaturation temperatures was performed as a test for size differences.

The consequences of heating DNA at temperatures below those at which DNA melts have been shown to depend on pH (ROGER and HOTCHKISS 1961) and upon

the way the DNA is prepared (GUINOZA and GUILD 1961). Simple inactivation behavior is obtained on DNA deproteinized by chloroform and octyl alcohol only if the pH lies below neutrality. Accordingly, the experiments reported here were done at pH 5.4 (0.02 M phosphate buffer). To protect against strand separation, the molarity of NaCl was raised to 1 M. Wild-type DNA bearing the *str-r41* gene was heated at 83° C, and samples removed at various times. Remaining biological activity was titered on a variety of *amiA* mutant recipient strains. In other experiments, DNA bearing gene *str-r41* and an *amiA-r* mutant gene was heated, and surviving activities titered on the wild-type strain.

Various HE and LE sites were examined to see whether they show any differences in subcritical heat inactivation rate. No significant differences were observed for the reference gene *str-r41* and HE sites *s22* and *s1*, on the one hand, and LE sites *s6*, *s4*, and *s17*, on the other. HE site *s29* is, possibly, slightly more resistant to subcritical heating than the reference gene *str-r41*. Subcritical heat damage does not, however, affect differentially HE and LE mutants.

The mapped sites *30*, *5* and *17* provide an occasion to test the validity of subcritical heat inactivation as a measure of marker size. Inactivation of *s30* or *r30*, as well as of *s17* and *r5*, proceed at the same rate. Since the multisite mutant *r30* and its *s* allele have the same sensitivity to subcritical heating as the sites with which it shows no recombination, it is doubtful that subcritical heat inactivation is really a sensitive measure of the dimensions of mutant sites. The inactivation curves for these sites are shown in Figure 4.

Further doubt is cast on the general usefulness of subcritical heating to measure dimensions of mutant marker genes by the fact that the *str-r41* gene is itself a multisite mutant of very appreciable dimensions (RAVIN and DE SA 1964). Yet, it proves to have the same resistance as virtually all of the *amiA* genes examined, which, with one exception, map as nonoverlapping mutants in the most intensely marked segment of DNA thus far known in a transformation system.

Finally, it should be noted that the multisite mutation *r30* (or its allele *s30*) is more, not less, readily integrated than the sites *r5* and *r17*, which *30* covers. This indicates that there is no simple relationship between efficiency and linear dimensions of genetic markers, just as there is no simple relationship between subcritical heat inactivation and efficiency.

Proximity to an end, and molecular weight heterogeneity of DNA as possible causes of efficiency differences: The possibility that our LE genetic markers are situated on DNA fragments of low molecular weight and therefore are less well absorbed by competent cells, is excluded by the results of crosses of the individual mutants by a single DNA preparation, wild-type for the *amiA* cistron. Since all of the *amiA* markers are linked, the same kind of DNA particle clearly is responsible for transformation of the various sites back to wild type, be they HE or LE.

While the mapping data do suggest that there is no obvious relationship between position of a mutant site within the cistron and its efficiency, a possible way of retaining the "proximity to an end" hypothesis as an explanation of low

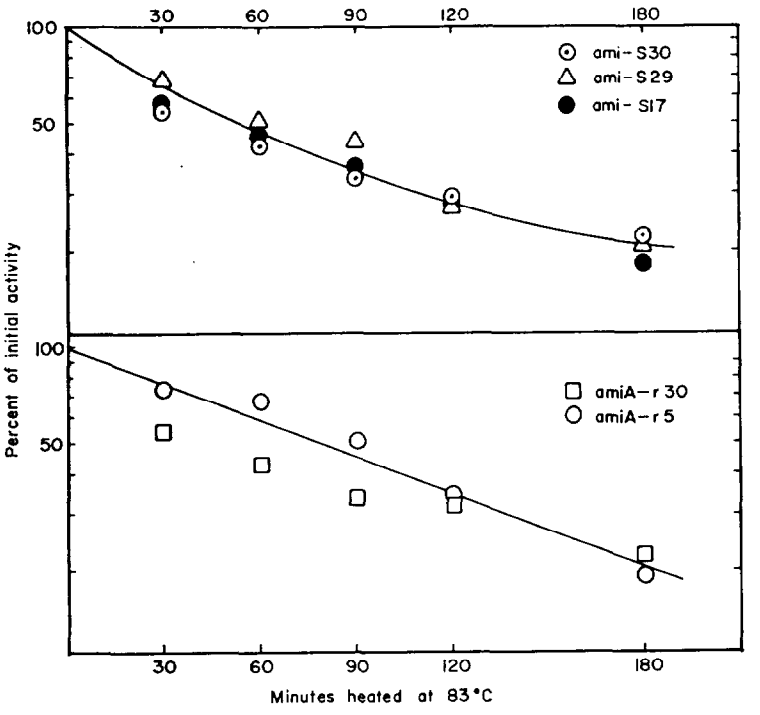

FIGURE 4.—Subcritical heat inactivation of mutant *r30*, its *s* allele, and *r* or *s* alleles of the sites *r30* covers. Upper curve, *s30* and *s17*. Lower curve *r30* and *r5*. In the upper curve, data on HE site *s20* are included for comparison.

efficiency has occurred to us. Were the pneumococcal chromosome circular, and were a mutational event leading to breaking the circle not lethal, one could suppose that LE mutations are always associated with a chromosomal break, while HE mutations are not. Thus, each LE mutant would be adjacent to an end of the chromosome, and would be near the end of a DNA molecule upon breakdown of the chromosome during the DNA extraction procedure. Further, when a LE mutant is serving as recipient cell in transformation, recombination could be strongly influenced by proximity of the mutant site in the recipient cell to the chromosomal break. One could, in this way, have LE mutants at any point of the cistron, which would show equally low efficiency in both directions of the crossing. The presence of a break near each LE mutant would not disturb the relative positions of the sites on the map, although it might disturb mapping with two-point crosses if sites of a cross were far enough apart to have been separated by a break. Since mapping has proven possible only when the sites in two-point crosses are quite close, recombination data alone could possibly not reveal the breaks. Another test of the "proximity to an end" hypothesis has thus been sought.

Ultraviolet light (UV) inactivates genetic markers when DNA is exposed *in vitro* (LATARJET, REBEYROTTE and DEMERSEMAN 1957; LERMAN and TOLMACH

1959; LITMAN and EPHRUSSI-TAYLOR 1959). It has been previously observed that various pneumococcal markers used in our laboratory (*str-r41*, *amiA-r1* and *opt-r2*) have different UV sensitivities (LITMAN and EPHRUSSI-TAYLOR 1959). The first two mutants, both highly efficient as transforming factors, are UV resistant, while the last mutant, showing a low efficiency, is UV sensitive, as shown in the curves of LITMAN and EPHRUSSI-TAYLOR. Marked differences in UV inactivation rates have been reported by others, working with transforming DNA in several bacterial systems. Possible explanations of differing sensitivities to UV have been enumerated and discussed (MARMUR *et al.* 1961). In personal discussions, LERMAN has suggested that genes near the end of a DNA molecule might be expected to be more resistant to the inactivating effects of UV irradiation, owing to the loss of excitation energy at the end of the molecule. This hypothesis was invoked to explain differences in rates of UV inactivation of different genetic markers in transforming DNA. In view of recent work on the mode of action of UV on transforming DNA (SETLOW and SETLOW 1962) and in particular the demonstration that dimerization of excited thymine residues is an important cause of inactivation, we can assume a somewhat different form of LERMAN's hypothesis insofar as this type of lesion is concerned, namely, that thymine residues in terminal sequences give rise more readily to dimers, owing to the possibility of easy strand separation and rotation of single strands at the ends of the molecule. Other suggested causes of different inactivation rates are: difference in target size of individual markers; differences in sizes of the molecules on which the markers are located; different localized sequences of bases in the DNA molecules which undergo photochemical changes with greater ease (MARMUR, *et al.* 1961). Clearly, by studying the UV sensitivity of a significant number of closely linked mutant sites, several of these possible explanations can be eliminated. In the only reported study which approaches this end, the pair of linked markers *ery-r2 ery-r3* was examined (MARMUR, *et al.* 1961). It was concluded that the greater UV sensitivity of the *ery-r3* marker relative to the *ery-r2* is not due to differences in molecular weight of the DNA molecules involved, since both were on the same molecule. On the other hand, thermal stability at subdenaturation temperatures indicated a greater dimension of marker *ery-r3*. There remained, thus, in the study of MARMUR, *et al.* (1961), three plausible explanations of greater UV sensitivity of the *ery-r3* marker: greater target size, special local base composition favoring photochemical damage, and proximity to an end.

The series of mutants shown in Figure 2 offers a unique occasion for testing the correlation between UV sensitivity and the efficiency of a transforming factor, under conditions in which we shall be able to narrow down the causes of UV sensitivity to a single cause: namely, highly localized factors which can, in last analysis, only be structural features in the neighborhood of the genetic markers in question, or of the markers themselves.

By irradiating with UV dilute solutions of the DNAs from various strains and titering residual activity of both *amiA-r* markers and the reference marker *str-r41* in these solutions, it has been possible to show a perfect correlation be-

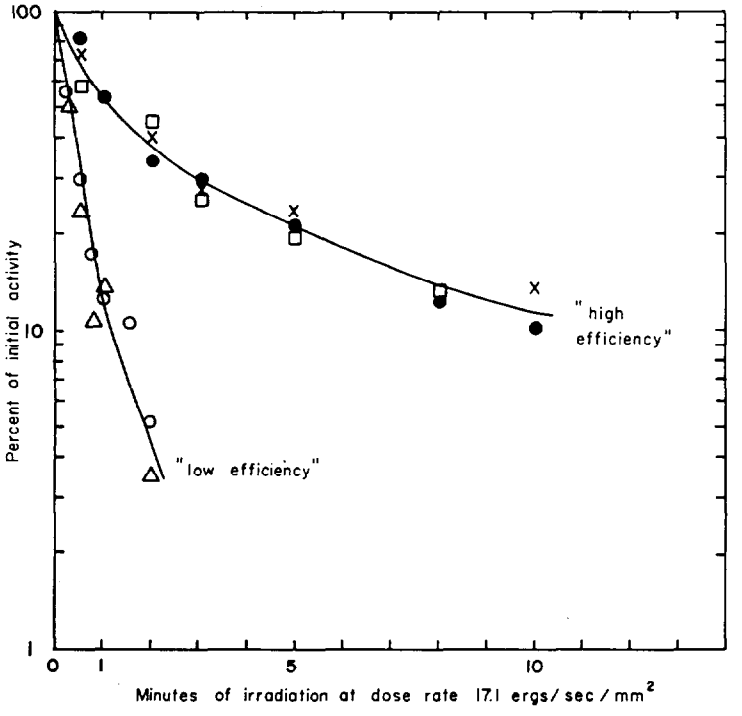

FIGURE 5.—Relative rates of inactivation of HE and LE mutants by UV irradiation.

tween UV sensitivity and efficiency of *amiA* marker genes in transformation. Five HE markers examined (*amiA-r1*, *r2*, *r19*, *r22*, and *r36*) showed a sensitivity to UV similar to that of the relatively resistant reference gene *str-r41*. Seven LE genes (*amiA-r21*, *r13*, *r17*, *r6*, *r26*, *r46*, and *r53*) showed in every instance a much greater sensitivity to UV inactivation. Figure 5 shows several of the inactivation curves observed.

We have excluded above the possibility that efficiency differences are caused by the *amiA* genes being on DNA fragments of different size, and shown that it is unlikely that they have widely different linear dimensions, both of which structrual features have been invoked to explain not only efficiency differences, but also increased UV sensitivity of transforming factors. In view of the high degree of correlation just demonstrated between UV sensitivity and efficiency we can conclude that the UV sensitivity and efficiency of the *amiA* mutants are determined by the same structural feature of the DNA, and that this feature is neither molecular weight heterogeneity, nor differences in linear dimesions.

If we retain the idea that low efficiency and UV sensitivity are correlated with proximity of the marker site to an end of a DNA molecule, we can suppose that greater UV sensitivity is due either (*a*) to higher yield of damage per photon absorbed near the end of DNA molecules, or (*b*) to a greater effect of a damaging hit on recombination, for marker genes near an end. As mentioned already, transformation of mutant cells to wild type can be performed and scored quanti-

tatively, just as well as transformation of wild type to mutant. LE mutants are spread throughout the *amiA* cistron, and in order to invoke proximity to an end as a cause of low efficiency, it was necessary to suppose that in each instance the event leading to mutation caused a break in the DNA, near each mutated site. However, the same sites in wild-type DNA should not all be proximal to breaks. (If the DNA is broken randomly upon extraction all marker genes have equal probability of being near an end. If it is broken nonrandomly, a restricted number of sites would be near ends.) We can, thus, test whether the wild-type alleles of LE mutations show high or low sensitivity to UV irradiation. To do this, it is enough to irradiate a single DNA preparation, wild-type for *amiA* but bearing *str-r41*, and follow the inactivation of normal *amiA-s* alleles by titering on the corresponding *amiA-r* strains. If proximity of a particular mutant site to an end is the cause of low efficiency and of UV sensitivity, then we would expect its wild allele to be UV resistant, although showing low efficiency when tested on a recipient strain bearing the mutant allele (because of proximity to a break in the chromosome of the recipient cell).

UV inactivation curves have been determined for the wild-type sites corresponding to a variety of mutant sites within the *amiA* cistron by the UV tech-

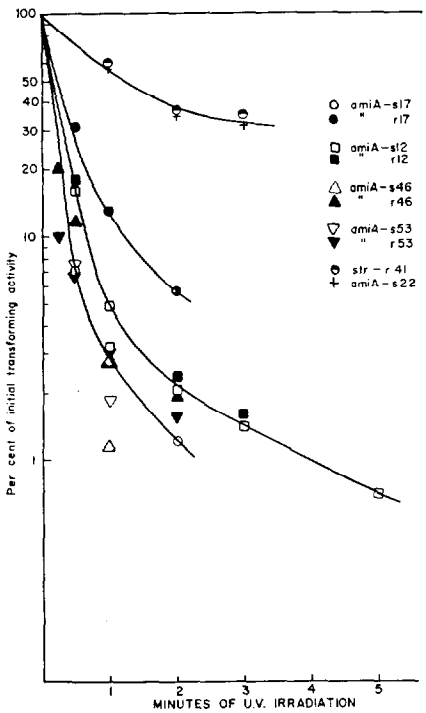

FIGURE 6.—Relative rates of inactivation of *s* alleles of HE and LE classes, by UV irradiation. The curves for both *s* and *r* alleles are shown for the LE genes.

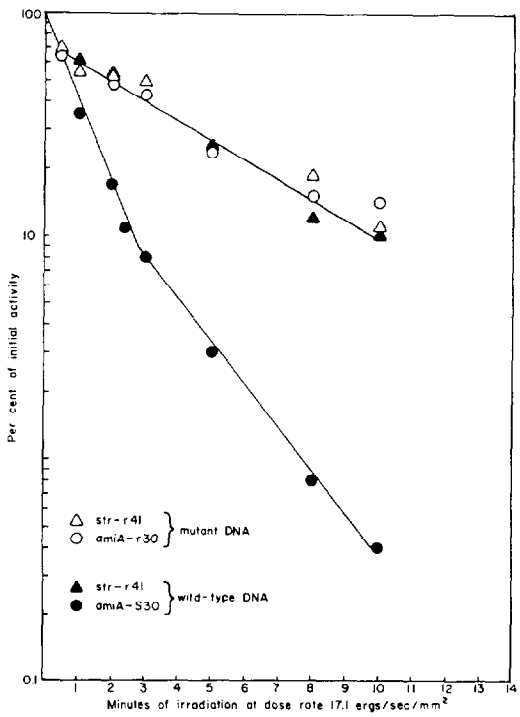

FIGURE 7.—Relative rates of inactivation of *r* and *s* alleles of multisite mutant *30*, compared with the reference gene *str-r41*.

niques used above, using synthetic medium supplemented with isoleucine for scoring *amiA-s* transformants in the *amiA-r* recipient populations. Some of the results of such a study are shown in Figure 6; the curves for each HE marker tested, *str-r41*, *amiA-s1*, *amiA-s19*, *amiA-s22* and *amiA-s36*, are essentially the same and all are equivalent to the inactivation curves for the corresponding mutant sites. Of the LE marker genes tested, *s12*, *s17*, *s46* and *s53*, site *r17* seems to be slightly more resistant than its *s* allele. Otherwise *r* and *s* alleles show essentially the same sensitivity to UV. From the fact that both mutant and wild-type alleles of the LE mutants tested are UV sensitive, it may be concluded that the "proximity to an end" hypothesis is not readily tenable for explaining low efficiency and UV sensitivity of the LE mutants in the *amiA* cistron.

UV sensitivity of gene amiA-r30 *and its wild-type allele:* The characteristics of gene *r30* are that it maps as a multisite mutant, and that it shows relatively high efficiency as a transforming factor, in spite of covering two LE sites. With respect to UV irradiation, the mutant allele shows a sensitivity identical to that of the *str-r41* gene, while its wild-type allele shows the sensitivity of a typical LE site (Figure 7). This is the first instance in which a great difference has been found between a mutant site and its wild-type allele. The sensitivity difference is the inverse of that postulated if mutation were to create a break in the DNA molecule adjacent to the mutant site. These results are compatible, however, with mutation *r30* being a deletion: the *r* mutant would have virtually no linear dimension, and have lost a segment of DNA particularly sensitive to UV (sites *5* and *17*). Its wild-type allele would have appreciable linear dimension, and contain a UV-sensitive segment. Inactivation of *r30* would be due to narrowing, by UV damage, of the zone within which switching must occur during recombination, according to the model of STAHL (1959), whereas inactivation of *s30* would occur, in addition, through hits in the UV-sensitive segment corresponding to the *r30* deletion.

DISCUSSION

The experiments described show that the efficiency with which a given genetic marker is transferred to, or transcribed into a recombinant chromosome in transformation is locally determined. These results do not exclude the possibility that in some specific instances, gross structural features such as molecular weight heterogeneity, or position relative to the end of a DNA molecule, are operating to determine efficiency. However, the present investigation is the first designed specifically to explore factors determining efficiency, and has employed a genetic system possessing unusually advantageous features. It has failed to support the notion that either of the gross structural features just mentioned plays any role in determining differential efficiencies of various genetic sites in a given DNA preparation and has shown that efficiency is in all likelihood site-specific for each marker examined, and independent of the polarity of transformation cross. In the absence of any experimental proof that efficiency differences between individual genetic markers in the same DNA preparation can be influenced by gross struc-

tural features, the weight of existing evidence is in favor of efficiency being determined by local structural features.

The four most striking findings of the present study, which will now be discussed, are the following: (1) Mutants which do not show the characteristics of deletions or multisite mutants are distributed into two nonoverlapping classes with respect to efficiency: high (HE) and low (LE = 0.1 × HE). (2) A multisite mutation which covers two LE sites shows a relatively high efficiency. (3) Spontaneous mutation gives rise to mutants of both efficiency classes, while the mutagens thus far examined give rise only to LE mutants. (4) There is a strict correlation between UV sensitivity of a site and the efficiency with which the unirradiated site will be included in a recombinant chromosome through transformation.

The existence of two nonoverlapping efficiency classes into which our first 73 mutants fall can only mean that the recombination process by which donor sites are integrated into a recipient-cell genome is subject to discontinuous variation, under the control of localized structural conditions. *amiA-r30*, the one proven multisite mutant, shows the lowest efficiency of the HE class. It covers two recombining LE sites, which are also UV-sensitive sites. From the fact that wild-type site *30* is UV sensitive, while mutant site *30* is UV resistant, one can infer that mutant *r30* is a deletion which eliminated a segment of UV-sensitive sequence. One arrives also at the conclusion that a deletion mutation makes a relatively efficient transforming factor. If one examines the efficiencies of the few mapped multisite mutants known for pneumococcus, notably the three reported by LACKS and HOTCHKISS (1960) in the amylomaltase locus, and the several mapped in the *str-r* locus by ROTHEIM and RAVIN (1961), ROTHEIM (1962) and RAVIN and DE SA (1964), no sharp discontinuity in the distribution of efficiencies is discerned: they are, relative to *str-r41*, 0.05, 0.13(2), 0.39, 0.41, 0.61, 0.87, 1.0. (It should be noted that *str-r41* is itself a multisite mutation). We are, therefore examining the hypothesis that mutants which do not clearly fall into either the HE or LE class are multisite mutants. Mutant markers *amiA-r32* and *r40* show efficiencies of over 1.6 in crosses with wild type, irrespective of the polarity of the cross. Whether they are multisite, or point mutations of a third efficiency class, or simply mutants falling in the extreme upper end of the HE distribution, remains to be discovered.

At present, however, we have only two clear efficiency classes into which our mutants fall. There are, in DNA, two types of base pairs. Is it possible that whenever a mutation is a transition of GC to AT it falls in one efficiency class, and whenever the mutation is an AT to GC transition it falls in the other? Both kinds of transitions would result in no hydrogen bond at the level of the mutated base pair in a paired structure composed of a single strand of DNA of the recipient cell, and the complementary single strand of the donor. One could raise the question whether, for instance, when an unpaired couple has G in the recipient strand and a mutant T opposite it in the donor strand, the T is rejected from the final recombinant structure more often than accepted, thus leading to

low efficiency for mutants which are GC → AT transitions. This model in its simplest form is excluded by the results of the reciprocal transformation experiments, which show that efficiency is independent of the direction of the cross. However, this type of model could be retained if we suppose instead that every time there is an unpaired G or C in the recipient strand, the recombination process excludes the donor base, whereas an unpaired A or T leads either to no bias, or preferential inclusion of the donor base. (The choice of base pairs here is arbitrary.)

The next most obvious "highly localized structural feature" which could determine efficiency is the composition of the base sequence adjacent to the mutation. Let us consider for a moment whether our present information furnishes a clue as to what sequence(s) might give rise to LE mutants.

If one tries to construct an argument on the basis of the fact that the two mutagens successfully employed, HNO_2 and EMS, give rise only to LE mutants, several difficulties are encountered. The first is that the chemical action for neither agent is so unique and specific that their mutagenic effects can be attributed to known chemical modifications of DNA. The one feature common to the two mutagens is that under the conditions in which they are active, guanines are altered and eventually hydrolyzed off of the polymer chain (LITMAN 1961; LAWLEY 1961). If the removal of guanine (leading either to a transition or a point deletion, for instance) were the cause of induction of mutations by these two agents, one could advance the proposition that such mutations are frequent in GC tracts, and that GC tracts of the donor are selectively rejected by the recombination process, causing low probability of inclusion of the mutation into the recombinant chromosome. Here a second difficulty arises. While the action of the mutagens on guanine may be the sole mutagenic event, there is no known reason why a GC pair adjacent to AT base pairs should not be affected some of the time by the mutagen. Knowing what kind of chemical change is mutagenic is not enough. What we need also to know is the nature of the base pairs adjacent to the altered base.

There is a strong correlation between great UV sensitivity and low efficiency. This means that the special structural features leading to low efficiency also lead to great UV sensitivity. UV irradiation of DNA causes thymine dimer formation, and this has been shown to account for about 70% of the inactivation of two different genetic markers in DNA of Hemophilus, at high UV doses (SETLOW and SETLOW 1962; 1963). The two genes examined by the SETLOWS showed somewhat different UV inactivation rates. Therefore, there is an indication from their studies that sensitivity differences of genetic markers are due to photochemical damages other than thymine dimer formation. However, nothing is known of the genetic complexity of the two Hemophilus markers examined, or whether gross structural factors are influencing their UV sensitivities. A detailed study of the mapped *amiA* genetic markers in pneumococcus, using the techniques of SETLOW and SETLOW for evaluating the fraction of damage due to thymine dimer formation, would provide more crucial evidence concerning the chemical basis of different UV sensitivity of transforming factors. We have attempted such a

study using yeast photoreactivating enzyme to destroy thymine dimers (WULFF and RUPERT 1962) but have encountered complications. Photoreactivating enzyme prepared by us from Baker's yeast contains a factor which inactivates unirradiated DNA, and which is partially inhibited by visible light. No clear experimental results on UV damaged DNA can be expected under the circumstances. It will be necessary either to purify the enzyme preparation or resort to monochromatic UV, in order to evaluate the role of thymine dimer formation in inactivation of our LE and HE mutations.

One can well ask, however, whether the different UV sensitivities of high and low efficiency mutants cannot be explained after all on a target-theory basis, rather than by supposing special base sequences to be particularly UV sensitive. Were the genetic integration of LE marker genes to proceed obligatorily through the insertion of relatively long segments of DNA, while that of HE genes primarily through the insertion of short sequences, the observed UV inactivation results would be explained. One would have to assume as a correlary that depurination does not affect the recombination process, since subcritical heating yields no differential inactivation of HE and LE sites. This hypothesis is the equivalent of saying that there are two qualitatively different recombination processes: the one, the rarer event, for the insertion of long segments of donor DNA; the other, a frequent event, for the insertion (or transcription) of short sequences of donor DNA into recombinants. The decision as to which process will be successful in inserting a particular mutant could depend on the base composition around the mutant, or the structure of the mutant itself (transition, transversion, point deletion). Since HE site *r30* and LE site *r53* are exceedingly close together on the map, both processes would have to be able to occur in this region, but in a mutually exclusive fashion. The frequent process can be visualized as a rapid "switching" process which selectively eliminates LE sites from the recombinant structure. Only when the rarer, qualitatively different process occurs would LE sites be inserted into a recombinant. This is a hypothesis that can be tested by genetic experiments, which are now under way.

If efficiency and UV sensitivity are determined primarily by the base composition adjacent to the mutant site, then, clearly, another correlation can be proposed; namely, that EMS and HNO_2 are mutagenic only when they alter a base adjacent to the particular sequence responsible for both UV sensitivity and low efficiency. It is quite clear from the work of CHAMPE and BENZER (1962) that base-analogue and other chemical mutagenesis in double stranded DNA is a far more complicated process than is often supposed. They attempted to identify the base transitions involved in a large number of mutations, induced in a variety of ways, by measuring the ability of mutants to revert under the influence of base analogues and hydroxylamine. Their rather low degree of success in obtaining clear identification revealed the complexity of mutagenesis, and led CHAMPE and BENZER to suggest that composition of base sequence adjacent to an altered or substituted base might be playing a role in the mutation process. Thus, our suggestion that EMS and HNO_2 cause mutations only when they alter a base adjacent to a particular base sequence finds ample echo in the experiments cited.

If, for example, the alteration of a base adjacent to A-T repeats (or G-C repeats) were to lead to a transition, then it need not matter which particular base be altered by the mutagen. We could then accept the prevailing evidence that HNO_2-induced mutation is caused primarily by alteration of cytosine and adanine (SCHUSTER and VIELMUTTER 1961; LITMAN 1961; WITTMANN 1960; WITTMANN and WITTMANN-LIEBOLD 1963), while that of EMS is due to alteration of guanine (LAWLEY 1961).

An alternate explanation of efficiency differences can be advanced, but it seems less likely than that just discussed. This is that the mutational event itself creates, or not, an incompatibility between donor and recipient DNAs. An inversion, or a very small deletion might, for example, cause pairing difficulties. The reason for considering this explanation less likely is that all of the mutations induced by HNO_2 and EMS, and half of the spontaneous mutations would have to be of this class. This seems a very high proportion of the total mutants examined. In other organisms, an appreciable number of HNO_2-induced mutations have, indeed, proven to be missense mutations resulting from single base transitions (TSUGITA and FRAENKEL-CONRAT 1960; TSUGITA 1962; WITTMANN 1960; WITTMANN and WITTMANN-LIEBOLD 1963), causing single amino acid substitution in proteins, not deletions or gross alterations of base sequence.

Differences in the ease with which highly localized points of a chromosome can participate in recombination are not generally recognized in genetic systems other than transformation and transduction. That such differences probably do exist, however, has already been pointed out by EPHRUSSI-TAYLOR (1961). This question will be re-examined in connection with the presentation of the results of two-factor crosses, in the report to follow.

The possibility that we may be able to recognize deletions on the basis of (a) their transforming efficiency when in donor DNA and (b) differential UV sensitivity of the deletion and its wild-type allele, is one promising perspective opened up by the present study. The possibility that one may be able to map the position of a particular type of base-sequence simply by mapping LE and HE mutants is another. We are, therefore, making every effort to determine the precise causes of high and low efficiency of transforming factors.

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SUMMARY

A group of 73 mutants in a single functional unit which determines resistance to aminopterin and sensitivity to an imbalance of branched amino acids distributes into two nonoverlapping classes with respect to the efficiency with which they are integrated into recombinant cells. High and low efficiency differ by a factor of about 10. Spontaneous mutants distribute equally between the two classes. Ethyl methane sulfate and HNO_2 induce only low efficiency mutants.

Thirty-nine of the mutants have been mapped at 30 sites, and while the remainder have not yet been completely mapped, it is clear that they lie at a number of additional sites. There is no simple relation between position on the map and efficiency. Independent mutations at the same site, yielding nonrecombining mutants, are thus far all of the same efficiency class. Efficiency is the same in both directions of transformation: mutant cells by wild-type DNA or wild-type cells by mutant DNA. Existing reversion data suggest that neither class can be described as deletions. Genetic evidence indicates therefore, that efficiency is site-specific, and determined by highly localized factors.

The consequences for biological activity of damaging transforming DNA *in vitro* have been examined, studying effects on mutant and wild-type alleles at the same site, and at different sites representing the two efficiency classes. Subcritical heat inactivation (depurinization) affects identically all marker genes tested, be they at high or low efficiency sites, mutant or wild-type. The one multisite mutant thus far found, *r30*, is inactivated by depurinization at the same rate as two recombining alleles it covers. Doubt is thus cast on subcritical heat inactivation as a sensitive measure of the dimensions of a mutant gene. UV irradiation has a strongly differential effect on mutants of the two efficiency classes: low efficiency genes are far more inactivated by low UV doses than are high efficiency genes. With one exception, UV sensitivity is shown to be very similar or identical for mutant and wild-type alleles at a given site. The one exception is mutant *r30* which is UV resistant, while its wild-type allele is UV sensitive. This is compatible with *r30* being a deletion of a base sequence responsible for both low efficiency and UV sensitivity. This sequence contains two low efficiency sites, 5 and *17*. UV sensitivity as well as low efficiency appear, both, to be site specific and caused by the same structural features of the DNA molecule, or of the recombination process.

On the basis of these observations the following hypotheses are advanced, and are currently under investigation: (1) That multisite mutations in general may be characterized by different UV sensitivities of the mutant and its wild-type allele. (2) That recombination in transformation proceeds through two distinct processes. In the one, responsible for genetic integration of high efficiency mutants, recombinants are constructed by a process in which rapid switching is occurring between donor and recipient DNA sequences. Low efficiency mutants are screened out of recombinants by this process, either because of special base composition in the region of the mutant, or because of the nature of the mutant (transversion, transition, point deletion). The second, rarer and qualitatively distinct process is presumed to involve the transfer to the recombinant of relatively long sequences of donor DNA, including both high and low efficiency sites.

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